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Research paper

Polymorphisms of *CYP2C8*, *CYP2C9* and *CYP2C19* and risk of coronary heart disease in Russian population

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ABSTRACT

Epoxyeicosatrienoic acids (EETs) are important vasoactive products of arachidonic acid metabolism with a wide range of biological actions in the cardiovascular system. The present study investigated whether single nucleotide polymorphisms (SNP) of genes coding cytochrome P450 2C subfamily, enzymes involved in biosynthesis of EETs, are associated with the risk of coronary heart disease (CHD). A total of 1255 unrelated Russian subjects comprising 561 patients with angiographically diagnosed CHD and 694 age- and sex-matched healthy subjects were included in the study. DNA samples from all study participants were genotyped for six common SNPs rs7909236, rs1934953 of *CYP2C8*, rs9332242, rs4918758 and rs61886769 of *CYP2C9* and rs4244285 of *CYP2C19* using by the Mass-ARRAY 4 system. SNP rs4918758 of *CYP2C9* was associated with decreased risk of CHD (codominant model) at a borderline significance with odds ratio adjusted for sex and age 0.61 (95% CI: 0.41–0.92, $P = 0.038$, $Q = 0.20$). SNP rs9332242 of *CYP2C9* showed a trend towards association with increased CHD risk in cigarette smokers ($P = 0.049$, $Q = 0.29$). Log-likelihood ratio test (LRT) pointed out epistatic interactions between rs9332242 and rs61886769 of *CYP2C9* (codominant model, $P_{interaction} = 0.02$), however, this *P*-value did not survive after correction for multiple tests. Bioinformatic analysis revealed a regulatory potential for a majority of the investigated SNPs. Our preliminary results demonstrate that polymorphisms of genes encoding CYP2C subfamily represent potential genetic markers of CHD susceptibility. Further studies are required to substantiate the contribution of these genes to the disease risk.

1. Introduction

Coronary heart disease (CHD) is a common cardiovascular disorder (CVD), a leading cause of mortality and disability worldwide (Roger et al., 2012). CHD is the primary cause of death in the Russian Federation, accounting for 45.3% of total CVD direct health care costs (~$3.1 billion) in the country (Notzon et al., 1998; Kontsevaya et al., 2013). Coronary heart disease is thought to be complex multifactorial disorder resulting from the interaction between multiple genetic and environmental factors (Poulter, 1999; Arnett et al., 2007). Genome-wide association studies have identified a number of genes related with CHD susceptibility in different populations of the world and provided insights into the molecular basis of the disease (Nikpay et al., 2015; McPherson and Tybjaerg-Hansen, 2016). However, the effect sizes of the identified loci responsible for CHD susceptibility were for the most part modest

Abbreviations: AA, arachidonic acid; CHD, coronary heart disease; CI, confidence intervals; CVD, cardiovascular disorder; EETs, epoxyeicosatrienoic acids; FDR, false discovery rate; HWE, Hardy-Weinberg equilibrium; LRT, log-likelihood ratio test; OR, odds ratio; PCR, polymerase chain reaction; ROS, reactive oxygen species; SNP, single nucleotide polymorphism; TFBS, transcription factor binding site; UTR, untranslated region.

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and collectively explained only a small fraction of the overall heritability (Hartiala et al., 2017), dictating the need of further investigation for understanding disease pathophysiology.

Alterations in the metabolism of arachidonic acid (AA) and other polyunsaturated fatty acids are known to be involved into the pathogenesis of CHD (Theken et al., 2012; Bellien and Joannides, 2013). Epoxyeicosatrienoic acids (EETs) are important vasoactive products of AA metabolism with a wide range of biological actions in the cardiovascular system (Bellien and Joannides, 2013; Spiecker and Liao, 2005; Seubert et al., 2007; Spector and Kim, 1851). Activating upon endogenous stimuli and oxidized by cytochrome P450 family 2 (CYP2) epoxygenases, EETs realize their cardiovascular effects through activating receptor-mediated signaling pathways and ion channels and possess vasodilatory, angiogenic and anti-inflammatory properties in the heart, vasculature and kidney (Spiecker and Liao, 2005; Spector and Kim, 1851; Fleming, 2001; Zordoky and El-Kadi, 2010). The CYP2 epoxygenases are found in the heart, vascular smooth muscle and endothelial cells where they are directly are involved in the biosynthesis of EETs from AA (Spiecker and Liao, 2005; Spector and Kim, 1851; Fleming, 2001; Zeldin, 2001).

Pursuing an interest in important cardiovascular functions of EETs, polymorphisms of genes encoding cytochrome P450 family 2 members became attractive candidates for genetic association studies of coronary heart disease (Lee et al., 2007; Ercan et al., 2008; Börgel et al., 2008; Marciante et al., 2008; Rothenbacher et al., 2013; Tang et al., 2016). Although some studies have revealed a relationship between genetic variants of CYP2 enzymes and CHD risk, nevertheless, most of them have yielded controversial results, showing that the pathogenetic role of these genes needs be clarified by independent studies. No studies have been done so far to investigate the contribution of genes encoding cytochrome P450 CYP2C subfamily to CHD susceptibility in Russians. Therefore, the purpose of this study was to investigate whether common single nucleotide polymorphisms (SNPs) of the CYP2C genes are associated with susceptibility to coronary heart disease in Russian population.

2. Methods

2.1. Study participants

Written informed consent was obtained from each study participant and the study protocol was approved by Ethical Review Committee of Kursk State Medical University. A total of 1255 unrelated Russian subjects comprising 561 patients with coronary heart disease and 694 healthy controls were included in this study. All patients with CHD were enrolled from Cardiology Divisions of Kursk Regional Clinical Hospital and Kursk Emergency Hospital as well as from Regional Cardiovascular Centre during a period between 2012 and 2015. All recruited patients had clinical signs or a history of CHD (angina or myocardial infarction) and angiographically confirmed narrowing of the coronary vessels by at least 50% in one or more major coronary artery. None of the enrolled CHD patients had signs and/or histories of congenital heart disease, cardiomyopathy, malignancy, connective-tissue disorder, chronic inflammatory disease, liver or kidney disease. The control group included blood donors, healthy volunteers as well as hospital-based patients recruited from surgical, traumatic, infectious divisions of Kursk hospitals without cardiovascular and other chronic diseases. The control group was recruited over several periods in the framework of our previous studies (Polonikov et al., 2007; Polonikov et al., 2008; Polonikov et al., 2009; Bushueva et al., 2014; Polonikov et al., 2015; Polonikov et al., 2017). Demographic and clinical characteristics of the study subjects are listed in Table 1. As can be seen from Table 1, the group of CHD patients was matched to the control group on both sex and age ($P > 0.05$). A percentage of positive family history of CHD, hypertension, diabetes mellitus was significantly greater in the case group *versus* healthy controls. No differences were found between the groups regarding to other characteristics shown in Table 1.

Table 1
Demographic and clinical characteristics of the study patients.

Baseline characteristics	Controls, n = 694	CAD patients, n = 561	*P*-value
Age, mean ± standard deviation	60.8 ± 9.0	61.7 ± 9.3	0.08
Males, n (%)	355 (51.2)	313 (55.8)	0.10
Body mass index (kg/m^2), mean ± standard deviation	27.9 ± 7.4	28.4 ± 8.3	0.26
Hypertension, n (%)	0 (0.0)	455 (89.2)	–
Diabetes, n (%)	0 (0.0)	43 (8.3)	–
Smokers (ever/never), n (%)	246 (36.7)	202 (38.9)	0.43
Positive family history of CHD, n (%)	137 (21.9)	159 (31.5)	**0.0003**
Positive family history of hypertension, n (%)	63 (12.5)	164 (26.3)	< **0.0001**
Positive family history of diabetes, n (%)	23 (3.7)	75 (14.8)	< **0.0001**

Bolded is statistically significant *P*-value.

2.2. Selection of genes and SNPs

Genes encoding CYP2C subfamily were selected on the basis of their involvement in the biosynthesis of EETs using information available at the KEGG PATHWAY (www.genome.jp/kegg/pathway.html), Reactome Pathway (www.reactome.org) and PharmGKB (www.pharmgkb.org) databases. Six common polymorphisms such as rs7909236, rs1934953 of *CYP2C8*, rs9332242, rs4918758 and rs61886769 of *CYP2C9* and rs4244285 of *CYP2C19* were selected based on their known functional relevance and/or haplotype tagging properties. The functionality of the SNPs was assessed *in silico* by the SNP Function Prediction tool developed by Xu and Taylor (Xu and Taylor, 2009) and available online at the SNPinfo Web Server (https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm). Common SNPs such as rs11572103, rs11572080 and rs10509681 of *CYP2C8* as well as rs1799853 and rs1057910 of *CYP2C9* were not included in the study because they have not been confirmed to be associated with CHD risk by two independent studies (Kaur-Knudsen et al., 2009; Haschke-Becher et al., 2010). Among polymorphisms of the *CYP2C19* gene, the only SNP rs4244285 was selected for our study because it is the most common variant associated with the formation of a dysfunctional protein (Desta et al., 2002).

2.3. Genotyping

Genomic DNA was extracted by standard phenol/chloroform procedure from whole blood samples obtained from all study participants. Polymerase Chain Reaction (PCR) was performed on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, USA). SNP genotyping was performed using by the Mass-ARRAY 4 system (Agena Bioscience Inc., San Diego, CA, USA) at the Core Facility "Medical Genomics" in the Research Institute of Medical Genetics, Tomsk National Medical Research Center (Tomsk, Russia). Blind replicates were included for quality control.

2.4. Data analysis

An association analysis between SNPs and CHD risk could detect a difference of 4–7% in the genotype distributions between the cases and controls assuming 75–98% statistical power and a 5% type I error

($\alpha = 0.05$) on the basis of the sample sizes of 561 CHD patients and 694 healthy controls. Allele frequencies were estimated by the gene counting method, and the chi-squire test was used to assess significant departures from Hardy–Weinberg equilibrium. Categorical variables were also compared by using the chi-squire test. Allele, genotype and haplotype frequencies in the study groups were evaluated by SNPStats, software which has been designed to analyze genetic associations using SNPs (Solé et al., 2006). The association between genotypes and CHD risk was measured by multiple logistic regression analysis to calculate odds ratios (OR) with 95% confidence intervals (CI) and adjusted for age and gender at codominant genetic model. Statistical calculations were performed by using the SNPassoc package for R (González et al., 2007) and SNPStats software (Solé et al., 2006). Epistatic interactions between SNPs were analyzed by SNPassoc package for R using a log-likelihood ratio test (LRT) (González et al., 2007) and assuming codominant, dominant and recessive models. Haplotypes of *CYP2C8* and *CYP2C9* were estimated in entire groups of CHD patients and controls using by the SNPStats software. *P*-value ≤ 0.05 was set to be statistically significant. As an adjustment for multiple testing, false discovery rate (FDR) based *Q*-value was calculated for each SNP using the method proposed by Benjamini and Hochberg (1995) and implemented in the FDR calculator available online at http://www.sdmproject.com/utilities/?show=FDR. Significance of the associations was assessed by a 0.20 threshold of *Q*-value, as previously suggested (Smith et al., 2007).

The regulatory potential of the studied SNPs was evaluated by using the SNP Function Prediction tool (https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.php) (Xu and Taylor, 2009) utilizing an information of the TRANSFAC database on potential transcription factor recognition sites (BIOBASE Corporation, Wolfenbuettel, Germany). Only transcription factor binding sites (TFBS) whose core or matrix match score was impacted, or which were eliminated or created by variant sequences are considered to be regulatory within a particular SNP. The SNP Function Prediction tool was also utilized to scan the SNPs for the presence of potential binding sites for microRNAs (miRNAs). In addition, rSNPBase, a database of curated regulatory SNPs (http://rsnp.psych.ac.cn) (Guo et al., 2014) and functional *in vitro* studies available from the literature were used to analyze and interpret genotype-phenotype relationships.

3. Results

3.1. Association analysis between CYP2C gene subfamily and CHD risk

Table 2 shows the genotype and allele frequencies of CYP2C polymorphisms. Allele and genotype frequencies were compatible with those reported in other European populations (the 1000 Genomes Project, http://www.internationalgenome.org). The genotype distribution for majority of SNPs was consistent with the population being in Hardy-Weinberg equilibrium, HWE ($P > 0.05$). A significant departure of genotype frequencies from HWE was found only for SNP rs4244285 of *CYP2C19* ($P = 0.02$). As can be seen from Table 2, SNP rs4918758 of *CYP2C9* was associated with decreased risk of CHD at a borderline significance with odds ratio adjusted for sex and age 0.61 (95% CI: 0.41–0.92, $P = 0.038$, $Q = 0.20$). Gender-stratified analysis (Supplementary Table 1) revealed no differences in allele and genotype frequencies between the case and control groups in both males and females ($P > 0.05$).

3.2. Interactions between SNPs of CYP2C gene subfamily and CHD risk

Then log-likelihood ratio test was performed to look for epistatic interaction between SNPs that determine the susceptibility to CHD (Table 3). As can be seen from Table 3, SNP rs4918758 of *CYP2C9* ($P = 0.011$), rs9332242 of *CYP2C9* ($P = 0.039$), rs4244285 of *CYP2C19* ($P = 0.031$) showed the main effects on CHD risk (recessive genetic model), as determined by the LRT. In addition, the log-likelihood ratio test analysis identified epistatic interactions between rs9332242 and rs61886769 of *CYP2C9* (codominant model, $P_{interaction} = 0.02$). However, these *P*-values did not survive after correction for multiple tests.

Table 2
Genotype and allele frequencies for SNPs of the CYP2C gene family in CHD patients and healthy controls.

Gene, polymorphism	Genotype, allele	Controls, n = 694 n (%)[a]	CHD patients, n = 561 n (%)[a]	OR (95 CI)[b]	*P*-value	*Q*-value
CYP2C8, G > T (rs7909236)	G/G	422 (60.8)	316 (56.3)	1.00	0.19	0.29
	G/T	239 (34.4)	210 (37.4)	1.19 (0.94–1.50)		
	T/T	33 (4.8)	35 (6.2)	1.42 (0.86–2.34)		
	T	305 (22.0)	280 (25.0)	1.18 (0.98–1.42)	0.08	0.27
CYP2C8, T > C (rs1934953)	T/T	316 (45.5)	245 (43.7)	1.00	0.56	0.67
	T/C	314 (45.2)	255 (45.5)	1.04 (0.82–1.32)		
	C/C	64 (9.2)	61 (10.9)	1.24 (0.84–1.83)		
	C	442 (31.8)	377 (33.6)	1.08 (0.92–1.28)	0.35	0.39
CYP2C9, C > G (rs9332242)	C/C	557 (80.4)	458 (81.6)	1.00	0.10	0.20
	C/G	124 (17.9)	100 (17.8)	1.00 (0.75–1.34)		
	G/G	12 (1.7)	3 (0.5)	0.29 (0.08–1.02)		
	G	148 (10.7)	106 (9.4)	0.87 (0.67–1.14)	0.31	0.39
CYP2C9, T > C (rs4918758)	T/T	310 (44.7)	262 (46.7)	1.00	0.038	0.20
	T/C	302 (43.5)	257 (45.8)	1.01 (0.80–1.28)		
	C/C	82 (11.8)	42 (7.5)	0.61 (0.41–0.92)		
	C	466 (33.6)	341 (30.4)	0.86 (0.73–1.02)	0.09	0.27
CYP2C9, T > C (rs61886769)	T/T	451 (65)	377 (67.2)	1.00	0.67	0.67
	T/C	220 (31.7)	168 (29.9)	0.91 (0.72–1.17)		
	C/C	23 (3.3)	16 (2.8)	0.81 (0.42–1.57)		
	C	266 (19.2)	200 (17.8)	0.91 (0.75–1.12)	0.39	0.39
CYP2C19, G > A (rs4244285)	G/G	543 (78.2)	448 (79.9)	1.00	0.086	0.20
	G/A	130 (18.7)	106 (18.9)	1.00 (0.75–1.33)		
	A/A	21 (3)	7 (1.2)	0.40 (0.17–0.95)		
	A	172 (12.4)	120 (10.7)	0.85 (0.66–1.08)	0.19	0.38

[a] Absolute number and percentage of individuals/chromosomes with particular genotype/allele.
[b] Odds ratio with 95% confidence intervals adjusted for age and gender.

Table 3
Epistatic interactions between CYP2C family gene polymorphisms in CHD (gene-gene interactions are evaluated by SNPassoc package for R (González et al., 2007)).

SNPs	Genetic models	*CYP2C8* (rs7909236)	*CYP2C8* (rs1934953)	*CYP2C9* (rs9332242)	*CYP2C9* (rs4918758)	*CYP2C9* (rs61886769)	*CYP2C19* (rs4244285)
CYP2C8 (rs7909236)	Codominant	0.192	0.324	0.148	0.363	0.841	0.314
	Dominant	0.093	0.935	0.718	0.324	0.821	0.134
	Recessive	0.251	0.589	0.161	0.171	0.311	–
	Overdominant	0.238	0.223	0.513	0.369	0.893	0.109
CYP2C8 (rs1934953)	Codominant	0.690	0.564	0.202	0.552	0.797	0.309
	Dominant	0.408	0.542	0.318	0.437	0.767	0.203
	Recessive	0.727	0.307	0.183	0.226	0.785	–
	Overdominant	0.344	0.995	0.137	0.740	0.451	0.156
CYP2C9 (rs9332242)	Codominant	0.180	0.533	0.120	0.209	**0.020**	0.772
	Dominant	0.734	0.680	0.632	0.205	0.060	0.887
	Recessive	0.195	0.274	**0.039**	–	0.560	–
	Overdominant	0.884	0.979	0.926	0.595	0.067	0.687
CYP2C9 (rs4918758)	Codominant	0.246	0.659	0.349	**0.041**	0.211	0.724
	Dominant	0.715	0.636	0.839	0.503	0.104	0.541
	Recessive	0.259	0.378	0.168	**0.011**	0.281	0.305
	Overdominant	0.336	0.982	0.879	0.398	0.257	0.380
CYP2C9 (rs61886769)	Codominant	0.756	0.731	0.541	0.570	0.670	0.287
	Dominant	0.532	0.611	0.930	0.862	0.408	0.232
	Recessive	0.559	0.577	0.435	0.328	0.591	–
	Overdominant	0.594	0.973	0.553	0.179	0.518	0.126
CYP2C19 (rs4244285)	Codominant	0.270	0.652	0.092	0.437	0.535	0.099
	Dominant	0.756	0.643	0.586	0.709	0.417	0.526
	Recessive	0.286	0.361	**0.030**	0.215	0.550	**0.031**
	Overdominant	0.762	0.989	0.919	0.891	0.950	0.879

The upper part of the matrix contains the *P*-values for epistatic interactions evaluated by log-likelihood ratio (LRT) test. The diagonal contains the main effects *P*-values from LRT for each SNP. The lower triangle contains the *P*-values from LRT comparing the two-SNP additive likelihood to the best of the single-SNP models. Bolded are statistically significant *P*-values for SNP-SNP interactions. *P*-values are adjusted for age and gender. All *P*-values are not adjusted for multiple tests.

3.3. Analysis of haplotypes and linkage disequilibrium between SNPs

The patterns of estimated haplotypes and their frequencies in the case and control groups are shown in Table 4. Three haplotypes of the *CYP2C8* gene and four haplotypes of the *CYP2C9* gene with a frequency > 1% have been identified in the study patients. Haplotype frequencies of *CYP2C8* and *CYP2C9* were compared between CHD patients and controls using a chi-square test (Table 4). There was no significant difference in the haplotype distribution of *CYP2C8* and *CYP2C9* between the case and control groups ($P > 0.05$). Polymorphisms rs7909236 and rs1934953 of the *CYP2C8* gene were in positive linkage disequilibrium ($D' = 0.946$, $P < 0.0001$), indicating that the wild type allele at one site is more likely to be associated with the wild type allele at the other site. All three polymorphisms of *CYP2C9* were in a strong linkage disequilibrium to each other ($D' = 0.958–0.989$, $P < 0.0001$).

Table 4
Estimated haplotype frequencies in CHD patients and controls.

	Haplotypes[a]	Controls	CHD patients	OR (95 CI)[b]	*P*-value
SNPs G > T (rs7909236) and T > C (rs1934953) of *CYP2C8*					
1	G-T	0.6735	0.6551	1.00	–
2	T-C	0.2116	0.2407	1.17 (0.97–1.42)	0.11
3	G-C	0.1068	0.0954	0.92 (0.70–1.20)	0.52
Global haplotype association *P*-value: 0.29					
SNPs C > G (rs9332242), T > C (rs4918758) and T > C (rs61886769) of *CYP2C9*					
1	C-T-T	0.6598	0.6941	1.00	–
2	C-C-T	0.1431	0.1267	0.85 (0.67–1.07)	0.16
3	G-C-C	0.1003	0.0935	0.89 (0.68–1.17)	0.40
4	C-C-C	0.0896	0.0837	0.87 (0.65–1.17)	0.36
Global haplotype association *P*-value: 0.16					

[a] Rare haplotypes with frequency < 0.01 are not shown.
[b] Odds ratio with 95% confidence intervals adjusted for age and gender.

3.4. Analysis of SNP-smoking interactions

Table 5 shows the results of analysis for SNP-smoking interactions and their contribution to CHD risk at codominant genetic model. As can be seen from Table 5, SNP rs9332242 of *CYP2C9* showed a trend towards association with increased risk of coronary heart disease in cigarette smokers ($P = 0.049$, $Q = 0.29$). No SNP-smoking interactions were found for other polymorphisms.

3.5. Bioinformatic analysis for regulatory potential of SNPs

Results of bioinformatic analysis for the regulatory potential of the studied SNPs are shown in Table 6. The SNP Function Prediction tool allowed identifying putative transcription factor binding sites at SNPs rs7909236 of *CYP2C8*, rs4918758 and rs61886769 of *CYP2C9* as well

Table 5
SNP-smoking interactions and susceptibility to CHD.

Genotype	Smokers				Non-smokers			
	Controls, n (%)	CHD patients, n (%)	OR (95% CI)[a]	*P*-value	Controls, n (%)	CHD patients, n (%)	OR (95% CI)[a]	*P*-value
SNP rs7909236 of *CYP2C8*								
G/G	151 (61.4)	125 (61.9)	1.00	–	256 (60.2)	165 (52.1)	0.83 (0.58–1.18)	NS
G/T	87 (35.4)	66 (32.7)	0.93 (0.62–1.39)	NS	145 (34.1)	131 (41.3)	1.17 (0.80–1.71)	NS
T/T	8 (3.3)	11 (5.4)	1.69 (0.66–4.34)	NS	24 (5.6)	21 (6.6)	1.11 (0.57–2.15)	NS
SNP × smoking interaction *P*-value: 0.21 (*Q*-value = 0.39)								
SNP rs1934953 of *CYP2C8*								
T/T	107 (43.5)	97 (48.0)	1.00	–	199 (46.8)	129 (40.7)	0.75 (0.51–1.12)	NS
T/C	118 (48.0)	89 (44.1)	0.83 (0.56–1.22)	NS	186 (43.8)	151 (47.6)	0.94 (0.64–1.39)	NS
C/C	21 (8.5)	16 (7.9)	0.85 (0.42–1.73)	NS	40 (9.4)	37 (11.7)	1.07 (0.61–1.85)	NS
SNP × smoking interaction *P*-value: 0.20 (*Q*-value = 0.39)								
SNP rs9332242 of *CYP2C9*								
C/C	204 (82.9)	155 (76.7)	1.00	–	333 (78.5)	267 (84.2)	1.10 (0.81–1.51)	NS
C/G	38 (15.4)	46 (22.8)	1.61 (1.00–2.60)	0.048	83 (19.6)	49 (15.5)	0.83 (0.53–1.31)	NS
G/G	4 (1.6)	1 (0.5)	0.31 (0.03–2.79)	NS	8 (1.9)	1 (0.3)	0.17 (0.02–1.35)	NS
SNP × smoking interaction *P*-value: 0.049 (*Q*-value = 0.29)								
SNP rs4918758 of *CYP2C9*								
T/T	111 (45.1)	87 (43.1)	1.00	–	189 (44.5)	154 (48.6)	1.09 (0.74–1.61)	NS
T/C	105 (42.7)	100 (49.5)	1.21 (0.82–1.80)	NS	187 (44.0)	139 (43.8)	1.00 (0.67–1.49)	NS
C/C	30 (12.2)	15 (7.4)	0.62 (0.32–1.23)	NS	49 (11.5)	24 (7.6)	0.67 (0.37–1.21)	NS
SNP × smoking interaction *P*-value: 0.52 (*Q*-value = 0.62)								
SNP rs61886769 of *CYP2C9*								
T/T	160 (65.0)	126 (62.4)	1.00	–	275 (64.7)	225 (71.0)	1.09 (0.78–1.54)	NS
T/C	78 (31.7)	68 (33.7)	1.10 (0.74–1.64)	NS	135 (31.8)	85 (26.8)	0.85 (0.57–1.26)	NS
C/C	8 (3.3)	8 (4.0)	1.22 (0.45–3.36)	NS	15 (3.5)	7 (2.2)	0.63 (0.24–1.61)	NS
SNP × smoking interaction *P*-value: 0.26 (*Q*-value = 0.39)								
SNP rs4244285 of *CYP2C19*								
G/G	192 (78.0)	162 (80.2)	1.00	–	336 (79.1)	248 (78.2)	0.92 (0.67–1.27)	NS
G/A	47 (19.1)	38 (18.8)	0.95 (0.59–1.53)	NS	76 (17.9)	64 (20.2)	1.07 (0.70–1.65)	NS
A/A	7 (2.8)	2 (1.0)	0.34 (0.07–1.66)	NS	13 (3.1)	5 (1.6)	0.47 (0.16–1.36)	NS
SNP × smoking interaction *P*-value: 0.75 (*Q*-value = 0.75)								

NS means non-significant.
[a] Odds ratio with 95% confidence intervals adjusted for age and gender (codominant genetic model).

as microRNA-binding sites and/or RNA binding protein mediated regulation sites at SNPs rs1934953 of *CYP2C8*, rs9332242 of *CYP2C9* and rs4244285 of *CYP2C19*. The lists of TFBS (rs4918758 and rs61886769 of *CYP2C9*, rs7909236 of *CYP2C8*) and microRNA-binding sites (rs9332242 of *CYP2C9*) are presented in Supplementary Tables 2–4 and 5, respectively.

4. Discussion

4.1. Summary of the study findings

To our knowledge, this is the first study investigated the contribution of SNPs rs7909236, rs1934953 of *CYP2C8* and rs9332242, rs4918758, rs61886769 of *CYP2C9* to the risk of coronary heart disease. We found that SNP rs4918758 of the *CYP2C9* gene is associated with decreased risk of CHD. The log-likelihood ratio test showed that SNP rs4918758 of *CYP2C9*, rs9332242 of *CYP2C9*, rs4244285 of *CYP2C19* have the main effects on CHD risk, and epistatic interactions between rs9332242 and rs61886769 of *CYP2C9* contribute to disease susceptibility. Moreover, SNP rs9332242 of *CYP2C9* showed a suggestive association with increased risk of coronary heart disease in cigarette smokers ($P = 0.049$, $Q = 0.29$). Bioinformatic analysis revealed the functionality of the studied SNPs: the presence of multiple putative TFBS at SNPs rs7909236 of *CYP2C8*, rs4918758 and rs61886769 of *CYP2C9* as well as microRNA-binding sites and/or RNA binding protein mediated regulation sites at SNPs rs1934953 of *CYP2C8*, rs9332242 of *CYP2C9* and rs4244285 of *CYP2C19*.

4.2. Pathogenetic relationship between SNPs and coronary heart disease

A literature search identified a few studies investigated the role of the CYP2C gene subfamily in the development of coronary heart disease (Lee et al., 2007; Ercan et al., 2008; Marciante et al., 2008; Rothenbacher et al., 2013). Numerous studies considered the studied

Table 6
Bioinformatic analysis for the regulatory potential of the studied SNPs in patients with coronary heart disease and healthy subjects.

SNP	Allele	Location	SNP function prediction (FuncPred)[a]		Regulatory annotations on SNPs (rSNPBase)[b]							Functional *in vitro* study of SNP [reference]
			TFBS	miRNA	rSNP	LD-proxy of rSNP ($r^2 > 0.8$)	Proximal regulation	Distal regulation	miRNA regulation	RNA binding protein mediated regulation	eQTL	
CYP2C8 (rs7909236)	G/T	Promotor	Yes	No	No	Yes	No	No	No	No	Yes	The variant allele T results in the creation of a CEBPalpha transcription factor consensus sequence, and is associated with increased transcription factor binding and promoter activity (Bahadur et al., 2002; Kirchheiner et al., 2008)
CYP2C8 (rs1934953)	T/C	Intron	No	No	Yes	Yes	No	No	No	Yes	Yes	ND
CYP2C9 (rs9332242)	C/G	3′UTR	No	Yes	Yes	Yes	Yes	No	No	Yes	Yes	ND
CYP2C9 (rs4918758)	C/T	5′UTR	Yes	No	Yes	Yes	Yes	No	No	No	Yes	Allele C is associated with decreased promoter activity as compared to allele T (Shintani et al., 2001; Cavallari et al., 2013)
CYP2C9 (rs61886769)	C/T	Promotor	Yes	No	No	Yes	No	No	No	No	No	ND
CYP2C19 (rs4244285)	A/C/G	Exon	No	No	Yes	Yes	No	No	No	Yes	No	A synonymous G > A transition in exon 5 that creates an aberrant splice site. This change alters the mRNA reading frame, which results in a truncated, non-functional protein (de Morais et al., 1994)

Data predicted by the SNP Function Prediction tool, National Institute of Environmental Health Sciences (https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.php): TFBS, transcription factor binding site; ND, no data.
Data obtained at rSNPBase, a database of curated regulatory SNPs (http://rsnp.psych.ac.cn): rSNP, rSNPBase identified regulatory SNPs; LD-proxy of rSNP ($r^2 > 0.8$), SNP in strong LD with rSNPs; proximal regulation, SNP involved in proximal transcriptional regulation; distal regulation, SNP involved in distal transcriptional regulation; miRNA regulation, SNP within mature miRNA; RNA binding protein mediated regulation, SNP involved in RNA binding protein-mediated post-transcriptional regulation; eQTL, SNP with experimental eQTL evidence.

genes as candidates for pharmacogenetic investigations of drugs (Hirota et al., 2013; Backman et al., 2016). Ercan with co-workers investigated SNPs rs1799853 (*CYP2C9*2*), rs1057910 (*CYP2C9*3*) and rs4986893 (*CYP2C19*3*) and found that cigarette smokers with heterozygote genotype for *CYP2C9*2* had 3.7-fold risk of developing coronary heart disease (Ercan et al., 2008). Additionally, the *CYP2C19*3* variant (SNP rs4986893 or rs57081121) was found to associated with a three-fold risk of coronary atherosclerosis (Ercan et al., 2008). Excepting participation in biosynthesis of EETs, CYP2C19 is also involved in the metabolism of a number of drugs such as platelet aggregation antagonists, proton pump inhibitors, anti-diabetes and anti-cancer drugs (Wei et al., 2008). SNP rs4244285 of *CYP2C19* (*CYP2C19*2* allele) represents a synonymous 681G > A transition in exon 5 that creates an aberrant splice site altering the mRNA reading frame and resulting in a truncated non-functional protein (de Morais et al., 1994). An apparent protective effect of this SNP against CHD risk observed in our study can be interpreted by that the loss-of-function variant of *CYP2C19* is related with decreased enzyme activity and therefore decreased production of reactive oxygen species (ROS) by this cytochrome P450. Notably, the similar protective effect of the *CYP2C19*2* variant was noted against the risk of tuberculosis (Backman et al., 2016). Meantime, our finding is inconsistent with the study results of Rothenbacher et al. (2013) who revealed that patients with stable CHD and homozygous for the *CYP2C19*2* loss-of-function gene have an increased risk for subsequent myocardial infarction during 8 year follow-up. This means that functional studies on this SNP are required to clarify this inconsistence.

We found that SNP rs4918758 may possess a protective effect against the CHD risk. Comparing the SNPs constituting each promoter variants of *CYP2C9* against the variants located within putative TFBS, only two polymorphisms rs4918758 and rs61886769 were found to be located within potential recognition sites for numerous transcription factors. In particular, transcription factors CDPCR3, FAC1, LUN1, OCT1, PPARA, SOX9 and Tal-1beta:E47 have been identified to co-regulate the promoter activity of *CYP2C9* (see Supplementary Tables 2 and 3). These TFBSs were highlighted because they reduced or increased significantly matrix match score by the variant allele, as reported in accordance with the TRANSFAC database. It is known from the literature that allele C is associated with decreased promoter activity as compared to allele T (Shintani et al., 2001; Cavallari et al., 2013). Hence, homozygous genotype CC may be related with decreased risk of CHD because a decreased promoter activity of *CYP2C9* in such individuals is capable to enhance ROS production and formation of oxidative stress, an important mechanism of atherosclerosis (Kondo et al., 2009; Li et al., 2014).

4.3. SNP rs9332242-smoking interaction and CHD risk

Lee with co-workers (Lee et al., 2007) observed that cigarette smoking may modify the relationship between common polymorphisms I264M (rs1058930) and K399R (rs10509681) of *CYP2C8* related with decreased enzyme activity (Gao et al., 2010) and the risk of CHD. An interesting finding of our study was a suggestive association between SNP rs9332242 of *CYP2C9* and CHD risk in cigarette smokers. No functional *in vitro* studies were undertaken to analyze the relationship between polymorphism rs9332242 and expression levels of *CYP2C9*. We cannot exclude that the functional effect of SNP rs9332242 can be explained by linkage disequilibrium with other SNPs: at least 58 SNPs are being in LD with rs9332242 ($r^2 > 0.8$) in accordance with the rSNPBase database (http://rsnp.psych.ac.cn/snp.do?snp=rs9332242). The SNP Function Prediction tool allowed identifying TFBS and microRNA-binding sites located at the rs9332242 polymorphism. In particular, we found that SNP rs9332242 *CYP2C9* is located within binding sites for miRNAs such as miR-1321, miR-143, miR-216a, miR-299-3p, miR-552 and miR-595 as well as within recognition sequences for numerous putative TFBSs (see Supplementary Table 5). miRNAs are short non-coding RNAs involved in post-transcriptional regulation of gene expression most commonly resulting in translational inhibition or destabilization of the target mRNA. In the light of interpretation of the SNP-smoking interaction, miR-143 and miR-299-3p attract significant attention with respect to a potential dependence of their expression on smoking exposure. SNP rs9332242 is located at 3′ untranslated region (UTR) of the *CYP2C9* gene within miRNA binding site for miR-143 which was known to be strongly up-regulated in tobacco smokers (Wang et al., 2015). It is also known that smoking may cause significant changes in the expression of miR-299-3p whose binding site comprises SNP rs9332242 (Guled et al., 2009). In addition, the study of Ercan with co-workers observed a synergic effect of tobacco smoking and SNP rs1799853 on the risk of coronary atherosclerosis (Ercan et al., 2008). Taken together these data point out that SNPs located within miRNA target sites (*e.g.* rs9332242 and rs1799853) may play a pivotal role in the pathogenesis of smoking-related diseases as miRNAs bind to 3′ UTR regions of genes, thereby regulating their expression (Momi et al., 2014). It is important to note that miR-552, another miRNA binding site located at rs9332242, possesses a dual inhibitory ability on the target genes at transcriptional and post-transcriptional levels. This feature was demonstrated by the study with effective inhibition of the *CYP2E1* gene (Miao et al., 2016) which, like CYP2C9, is also involved in the formation of EETs from arachidonic acid (Rifkind et al., 1995). Further studying polymorphism rs9332242 of *CYP2C9* and associated miRNAs in conjunction with the intermodulation between them upon smoking exposure may help to provide mechanistic insights into the mechanism by which the locus influences the risk of coronary heart disease in cigarette smokers. On the one hand, the synergic effect of smoking exposure and genotype C/G (rs9332242) points out that the G variant increases the activity of CYP2C9 enzyme potentiating a pro-oxidant action of cigarette smoke through enhanced generation of ROS, oxidative stress and vascular inflammation. On the other hand, loss-of-function effect of this SNP may be responsible for a deficiency of EETs which are known to be involved in endothelium-dependent vasodilation, endothelial survival *via* cytoprotective functions, thereby preventing apoptotic and oxidative vascular injury (Spiecker and Liao, 2005; Fleming, 2001). In any case, further studies are required to analyze the functional effects of the studied SNPs and to confirm our suggestions.

4.4. Study limitations

The results of this study should be interpreted in the context of some limitations. The associations of the *CYP2C9* polymorphisms with CHD risk were weak, sometimes even suggestive, demonstrating the discovery nature of our study. The study findings do not allow us to draw definitive conclusion on a comprehensive contribution of the studied genes to the development of coronary heart disease. We investigated a limited number of SNPs of *CYP2C* gene subfamily. Because of an insufficient number of patients in the study groups, we did not perform gene-smoking interaction analysis stratified by gender with an appropriate statistical power. Hence, the results of our study should be considered as preliminary and further investigation in larger population-based samples is required to address issues discussed above.

5. Conclusion and perspective

Nevertheless, the present study points out the polymorphisms of *CYP2C9* could be potential genetic markers of susceptibility to coronary heart disease. The relationship between polymorphisms of *CYP2C9* gene and CHD risk also demonstrates an importance of epoxygenase pathway of arachidonic acid metabolism for disease pathogenesis. Better understanding the relationships between altered *CYP2C9* gene expression and function in patients with coronary heart disease

may provide novel insights into disease pathophysiology and suggest specific targeted interventions that may expand the therapeutic options for cardiovascular disease in the future.

Author contributions disclosure

All authors provided critical review of the manuscript. A.P. and M.S.: designed the study, designed and carried out the statistical/bioinformatic analysis and wrote the manuscript; A.K.: clinical data collection and analysis, disease diagnosis; M.B.: DNA extraction, performed genotyping; S.S.: DNA extraction, performed genotyping; I.P.: DNA extraction, performed genotyping; A.B.: carried out the quality control of the data; K.V.: carried out the quality control of the data; V.S.: database creation, statistical analysis; O.B.: biological data collection and processing; M.C.: study phenotyping, data analysis.

Uncited references

Feng et al., 2012
Spiecker et al., 2004

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2017.07.004.

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